

Original citation:

Alves, Patrícia M., Al-Badi, Eide, Withycombe, Cathryn, Jones, Paul M, Purdy, Kevin J. and Maddocks, Sarah E.. (2018) Interaction between *Staphylococcus aureus* and *Pseudomonas aeruginosa* is beneficial for colonisation and pathogenicity in a mixed-biofilm. *Pathogens and disease*, 76 (1). fty003.

Permanent WRAP URL:

<http://wrap.warwick.ac.uk/98211>

Copyright and reuse:

The Warwick Research Archive Portal (WRAP) makes this work by researchers of the University of Warwick available open access under the following conditions. Copyright © and all moral rights to the version of the paper presented here belong to the individual author(s) and/or other copyright owners. To the extent reasonable and practicable the material made available in WRAP has been checked for eligibility before being made available.

Copies of full items can be used for personal research or study, educational, or not-for profit purposes without prior permission or charge. Provided that the authors, title and full bibliographic details are credited, a hyperlink and/or URL is given for the original metadata page and the content is not changed in any way.

Publisher's statement:

This is a pre-copyedited, author-produced PDF of an article accepted for publication in *Pathogens and disease*, following peer review. The version of record Alves, Patrícia M., Al-Badi, Eide, Withycombe, Cathryn, Jones, Paul M, Purdy, Kevin J. and Maddocks, Sarah E.. (2018) Interaction between *Staphylococcus aureus* and *Pseudomonas aeruginosa* is beneficial for colonisation and pathogenicity in a mixed-biofilm. *Pathogens and disease*, 76 (1). fty003. is available online at: <https://doi.org/10.1093/femspd/fty003>

A note on versions:

The version presented here may differ from the published version or, version of record, if you wish to cite this item you are advised to consult the publisher's version. Please see the 'permanent WRAP URL' above for details on accessing the published version and note that access may require a subscription.

For more information, please contact the WRAP Team at: wrap@warwick.ac.uk

Interaction between *Staphylococcus aureus* and *Pseudomonas aeruginosa* is beneficial for colonisation and pathogenicity in a mixed-biofilm

Patrícia M. Alves¹, Eide Al-Badi¹, Cathryn Withycombe¹, Paul M. Jones¹, Kevin J. Purdy², Sarah E. Maddocks¹

¹Department of Biomedical Sciences, Cardiff School of Health Sciences, Cardiff

Metropolitan University, Western Avenue, Llandaff, Cardiff, CF5 2YB, United Kingdom

²School of Life Sciences, University of Warwick, Coventry, CV4 7AL, United Kingdom

Correspondance : smaddocks@cardiffmet.ac.uk

Keywords : polymicrobial, wound-infection, biofilm, aggregation, invasion, adhesion

Abstract

Debate regarding the co-existence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* in wounds remains contentious, with the dominant hypothesis describing a situation akin to niche partitioning, whereby both microorganisms are present but occupy distinct regions of the wound without interacting. In contrast, we hypothesised that these microorganisms do interact during early co-colonisation in a manner beneficial to both bacteria. We assessed competitive interaction between *S. aureus* and *P. aeruginosa* in biofilm cultured for 24-72 h and bacterial aggregates

analogous to those observed in early (<24h) biofilm formation, and interaction with human keratinocytes. We observed that *S. aureus* predominated in biofilm and non-attached bacterial aggregates, acting as a pioneer for the attachment of *P. aeruginosa*. We report for the first time that *S. aureus* mediates a significant ($P<0.05$) increase in the attachment of *P. aeruginosa* to human keratinocytes, and that *P. aeruginosa* promotes an invasive phenotype in *S. aureus*. We show that co-infected keratinocytes exhibit an intermediate inflammatory response concurrent with impaired wound closure that is in keeping with a sustained pro-inflammatory response which allows for persistent microbial colonisation. These studies demonstrate that, contrary to the dominant hypothesis, interactions between *S. aureus* and *P. aeruginosa* may be an important factor for both colonisation and pathogenicity in the chronic infected wound.

Introduction

The prevention and management of biofilm in wounds is a priority for clinicians and researchers, and is allied with the increased global focus on antimicrobial stewardship due to the increasing number of multi-drug resistant bacteria (Mashburn et al., 2005; Tümmler et al., 2014; Barnabie et al., 2015; WUWHS, 2016). Biofilms are thought to be present in most wounds and are known to be comprised of multiple bacterial species; moreover, their incidence is documented as having a deleterious impact on wound healing.

There are a number of different guidelines supporting the diagnosis and treatment of biofilms in wounds, but detecting biofilm is challenging in clinical practice (Dohmen et al., 2008; Rhoads et al., 2008; Hoiby et al., 2010; Gottrup et al.,

2011; Metcalfe et al., 2016; WUWHS, 2016). Most guidelines are predicated on expert opinion, yet in reality little is known about the impact of bacterial interaction on the development and severity of co-infected wounds, from which *Staphylococcus aureus* and *Pseudomonas aeruginosa* are most commonly isolated (Serra et al., 2015).

An extensive body of evidence exists describing the relationship between *S. aureus* and *P. aeruginosa* using cystic fibrosis (CF) lung infection models (Baldan et al., 2014; Filkins et al., 2015; Maliniak et al., 2016). The lungs of children with CF are readily colonised by *S. aureus* during the early years of life, with colonisation by *P. aeruginosa* during mid to late teenage years. Colonisation with *S. aureus* is associated with a higher propensity for secondary colonisation with *P. aeruginosa*, and once present the latter rapidly establishes and eventually predominates (Ahlgren et al., 2015; Limoli et al., 2016). Co-infection with *P. aeruginosa* and *S. aureus* ultimately results in a poor clinical outcome for the patient correlated with increased inflammatory markers.

Less is understood about the relationship between *S. aureus* and *P. aeruginosa* in the chronic infected wound. Chronic wounds are often co-colonised by *S. aureus* and *P. aeruginosa*, and once contaminated with *P. aeruginosa* the infected wound becomes highly recalcitrant to treatment (Serra et al, 2015). Biopsies of established chronic infected wounds have indicated that *S. aureus* and *P. aeruginosa* exist apart in distinct, separate niches with *P. aeruginosa* found within the deeper tissues and *S. aureus* at the surface of the wound (Fazli et al., 2009). Hypotheses currently describing co-colonised wounds therefore reject the idea that they are truly polymicrobial based on this pattern of colonisation (Woods et al., 2012; Phalak et al., 2016).

Ecological theories of competitive exclusion and the niche concept support current observations of *S. aureus* and *P. aeruginosa* in chronic infected wounds (Davies, 2006). These principles state that two-species competing for the same resource cannot stably co-exist if other ecological factors are constant. Consequently, one of the two competitors will become dominant and drive the other either to extinction or another ecological niche. However, this is rarely observed in nature, where multiple species co-exist in a single environment.

We hypothesise that the niche partitioning observed in chronic wounds occurs over a long period of time, and these two pathogens initially co-exist and interact in a beneficial manner in much the same way as oral microorganisms in plaque development. Furthermore, we suggest that inter-bacterial interactions between *S. aureus* and *P. aeruginosa* impact upon temporal colonisation, pathogenicity, and inflammatory responses associated with infection.

Therefore the objectives of this study were to assess the relative competition between *S. aureus* and *P. aeruginosa* in co-cultured biofilm, and to determine whether co-culture resulted in synergy of growth and virulence concurrent with enhanced damage, diminished immune response and impaired repair of a human keratinocyte cell line.

Materials and Methods

Bacterial strains

Pseudomonas aeruginosa reference strain ATCC 9027 (NCIMB 8626) and *Staphylococcus aureus* NCTC 13142 (EMRSA-15) were used throughout the study. Both strains are associated with skin/wound infections. All strains were cultured aerobically at 37°C in nutrient broth (NB; Oxoid). Selective media were used to

recover the isolated from co-culture experiments, these were *Pseudomonas* Selective Agar (Sigma Aldrich, UK) and Baird Parker Agar (Sigma Aldrich, UK).

Preparation of bacterial extracts

Biofilms of *S. aureus* and *P. aeruginosa* were cultured in 5ml NB in sterile plastic Petri dishes for 24, 48 or 72h at 37°C, media was not changed during this time. Spent media (containing constituents secreted by biofilm bacteria) from these three time points was aspirated, centrifuged at 9,000 *g* and heat treated at 60°C for 1h. Cell extracts were prepared by washing the biofilm three times with phosphate buffered saline (PBS; Oxoid) scraping biofilm cells from the Petri dish using a cell scraper and re-suspending them in 1ml sterile PBS prior to heat treatment as described above. Secreted and cell extracts were stored at -20°C.

Static biofilm model

Co-cultured biofilms were prepared by culturing planktonic *P. aeruginosa* and *S. aureus* (separately) aerobically for 16h in NB at 37°C, which were subsequently harvested by centrifugation at 3,000 *g*, at 4°C for 5 minutes. Bacterial cell pellets were re-suspended in 1ml of PBS (Oxoid) and the optical density at 600nm (OD₆₀₀; SPECTROstar^{Nano}, BMG Labtech) of the culture was adjusted to 0.1 (\pm 0.05; equivalent to 1×10^8 cfu ml⁻¹); 100µl total volume of bacterial cells from both cultures were mixed in a 1:1 ratio and 5µl used to inoculate wells of a 96-well microtitre plate (MTP), containing 50µl NB per well. Following incubation at 37°C for 24, 48 or 72h, media and non-adherent cells were aspirated and the biofilm biomass was

determined by staining with 0.5% (w/v) crystal violet, which was re-solubilized with 7% (v/v) acetic acid and its concentration determined by its absorbance at 595nm.

For analysis of the effect of bacterial extracts on biofilm formation and disruption, 10µl of either secreted or cell extract was added to developing (at time zero) biofilm or pre-cultured (24h) biofilm. In the latter case, pre-cultured biofilm was incubated with the extract for 1h at 37°C prior to staining for biomass.

For sequential biofilm culture, single species biofilms were grown as described above and following 24, 48 or 72h incubation, the second microorganism was inoculated at 1×10^8 CFU ml⁻¹ and growth allowed to continue for a further 24h at 37°C. Biomass was assessed as described above.

Total viable count and assessment of viability

Biofilms were grown as described above. Media and planktonic cells were aspirated and the biofilms scraped off the surface of the plate using a cell scraper; 100µl of PBS was added to each well and the bacterial aggregates re-suspended by vigorous pipetting. Cell suspensions were transferred to sterile microcentrifuge tubes, vortexed to ensure the cell suspension was homogenous and, serially diluted from 10^{-1} to 10^{-7} using PBS. The Miles & Misra technique was used to determine the CFU ml⁻¹ and was performed in triplicate (Miles et al., 1938). Plates were incubated at 37°C for 24h.

Protein binding assay

To determine the adherence of co-cultured *S. aureus* and *P. aeruginosa* to immobilized fibronectin, fibrinogen and collagen (Sigma Aldrich) 1µg of each protein was used to coat the surface of a 96-well MTP; proteins were dissolved in coating

buffer (20mM sodium bicarbonate buffer, pH 9.3) and wells were blocked with 1% (w/v) bovine serum albumin (BSA) to prevent non-specific binding. *P. aeruginosa* and *S. aureus* were grown and a 1:1 ratio was prepared as previously described. Bacterial cells were adhered to protein coated wells for 2h at 37°C. Following incubation, non-adherent cells were aspirated from the wells; the plate was washed with PBS and adherent cells fixed with 25% (v/v in water) formaldehyde for 30min at room temperature. Adherent bacteria were stained with 0.5% (w/v) crystal violet which was then re-solubilized and analysed as described above. The assay included a control set of wells containing only coating buffer without protein ligands to determine whether the wells of the plate were sufficiently coated. The experiments used three biological replicates and each assay was performed in triplicate.

Assays to determine affinity of *S. aureus* and *P. aeruginosa* for bacterially-derived cellular extracts utilized the same protocol except that wells were coated with 50µl aliquots of extract diluted in an equal volume of coating buffer. Blocking assays were carried out as described above, however the single species biofilm was blocked by the addition of an anti-staphylococcal antibody (1:200 in PBS; ThermoScientific) for 1h at room temperature, prior to the addition of *P. aeruginosa*.

Aggregation assay

Bacterial suspensions and ratios were prepared as previously described, to achieve a final volume of 1ml in co-aggregation buffer (1mM Tris-HCl [pH 8], 150mM NaCl, 0.1mM CaCl₂·2H₂O, 0.1mM MgCl₂·6H₂O). Fibronectin, fibrinogen or collagen (final concentration 1 µg ml⁻¹) were added separately to the aliquot of bacterial cells. In an untreated control, the proteins were replaced with PBS to maintain the appropriate volume and concentration. Triplicate samples were thoroughly mixed and incubated

at 37°C. The OD₆₀₀ was measured after 210min. The composition of aggregates was determined by TVC, using the method of Miles and Misra, previously described. The experiments used three biological replicates and each assay was performed in triplicate.

Gentamicin protection assay

HaCaT (an immortalised keratinocyte cell line) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS) (Biosera, East Sussex, UK), 2mM glutamine and 2.5g/L glucose (all supplements were purchased from Gibco BRL, Paisley, UK). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ for optimal growth and proliferation.

The gentamicin protection assay followed the method of Rasigade *et al.* with some modifications (Rasigade et al., 2011). HaCaT cells were seeded at 50,000 cells/well in 24-well plates and incubated at 37°C with 5% CO₂ for 48h in culture medium, as described above. Bacterial strains were cultured and 1:1 ratios prepared as previously described (using DMEM). HaCaT cells were washed twice with DMEM and were infected with bacterial cells at a multiplicity of infection of 200:1. Cells were incubated at 37°C for 2h to allow for adhesion and internalization of bacteria, and then washed with PBS to remove any unbound bacterial cells. For adhesion assays, cells were osmotically shocked using pure water and extensively pipetted to release all cell-associated bacteria. For invasion assays, infected cells were incubated for a further 1h in culture medium containing 200 µg ml⁻¹ gentamicin to kill extracellular, but not internalized, bacteria. Cells were washed twice in PBS

and treated with pure water as described above to release internalized bacteria. For both assays bacterial cells were enumerated using selective media as described previously. The number of adherent bacteria was calculated by subtracting the number of internalized bacteria from the total number of cell-associated bacteria.

ELISA for pro-inflammatory cytokines

Cell culture media was harvested from scratch assays and analysed by ELISA to determine the concentration of IL-1 β , IL6 and TNF- α . These included untreated (control) cells, and these treated with bacterial extracts derived from single and two-species biofilms cultured for 24, 48 and 72h. ELISA's were carried out according to the manufacturer's instructions (Novex ELISA Kits; ThermoFisher Scientific). Briefly, 50 μ l of cell supernatant (or standard) was added to each well of a pre-coated 96well MTP followed by 50 μ l of biotinylated antibody reagent and incubated at room temperature for 3h. The plate was washed three times with wash buffer (provided by the manufacturer) and 100 μ l of Streptavidin HRP solution added and, the plate incubated at room temperature for 30min. The plate was washed three times and 100 μ l of developer added to each well. After 30min 100 μ l of stop solution was added to each well and the absorbance recorded at 450/550nm using a SPECTROstar^{Nano} reader (BMG Labtech).

In vitro scratch wound healing assays

HaCaT cells were seeded at 50,000 cells/well in a 12-well plate, and grown to 90% confluence prior to beginning scratch assays. Once confluence was reached media was removed and a scratch wound made using a 10 μ l pipette tip; each well was washed with warm PBS (37°C) and cells incubated for a further 24h in the presence and absence of secreted or cellular components derived from single or two-species

biofilms (as previously described). Images were taken at 24h to observe wound closure.

Statistical analysis

Statistical analysis of data used ANOVA followed by Tukeys post-hoc test, using Minitab v14.

Results

***S. aureus* predominates in early biofilm**

Early biofilm formation occurs via a combination of bacterial auto/co-aggregation, and attachment to a sub-stratum. Two-species biofilms comprised of *S. aureus* and *P. aeruginosa*, cultured for 24-72h, showed a statistically significant ($P < 0.05$) increase in the amount of accumulated biofilm (Figure 1A) when stained with crystal violet, relative to single species biofilms of *S. aureus*, cultured for the same amount of time (Figure 1B). However, the biomass increases observed between *P. aeruginosa* mono-species and dual-species biofilm, did not equate to a statistically significant fold-change when compared to single-species biofilms of *P. aeruginosa* over the same period (Figure 1B). Fold changes were calculated relative to single-species biofilms for each bacterium. Analysis of the composition of two-species biofilm by CFU (using selective media as indicated in materials and methods) and subsequent calculation of competitive relative index, indicated that *S. aureus* predominated at each time point with higher competitive relative indices than *P. aeruginosa* (Figure 1C). Competitive relative indices are derived from CFU and therefore higher competitive indices signify higher numbers of bacteria. This suggests that it is advantageous for *S. aureus* to be part of a two-species biofilm in terms of synergistic growth.

Early biofilm development for both *S. aureus* and *P. aeruginosa* relies on the formation of bacterial aggregates in suspension, which subsequently attach to a solid substratum (Birkenhauer et al., 2014). Relative to auto-aggregation of each bacterium alone, co-aggregation of both bacteria together in suspension, led to reduced total aggregation after 210 min, relative to *S. aureus* alone (auto-aggregated), but was not statistically different compared to *P. aeruginosa* alone (auto-aggregated) over the same time period (Figure 2A). By comparing the extent of co-aggregation to auto-aggregation, for each bacterium it was apparent that co-aggregation negatively impacted on *S. aureus* (i.e. the propensity to aggregate was less), but positively impacted on the propensity for *P. aeruginosa* to aggregate (i.e. *P. aeruginosa* aggregated more readily) (Figure 2A). Competitive relative indices derived from TVC analysis of bacterial aggregates indicated that despite the reduced aggregation observed for *S. aureus* when co-aggregated with *P. aeruginosa*, *S. aureus* comprised the largest proportion of the bacterial aggregates (Figure 2B).

Collectively these data indicate that during the early stages of biofilm development, *S. aureus* has a competitive advantage over *P. aeruginosa* in terms of aggregation, attachment and growth, but also promotes increased aggregation of *P. aeruginosa*. This could be advantageous since the latter has fewer known surface adhesins and attachment to *S. aureus* could provide a means of integrating into the competitive environment of the early biofilm (Prince, 1992; Clarke & Foster, 2006).

S. aureus early biofilm influences the attachment and maintenance of *P. aeruginosa* biofilm

Clinical analysis of wound infection shows a typical “Gram-negative shift” over time, (Alttoparlak et al., 2004; Dalton et al., 2011; Guggenheim *et al.*, 2011; Pastar et al.,

2013). We investigated whether Gram positive *S. aureus*, through provision of a conditioning layer, promoted this shift by enhancing attachment of *P. aeruginosa*, as is observed for other human biofilms (Kolenbrander et al., 2010). Using mono-species biofilm of each respective bacterium as a base-line for comparative biomass accumulation and fold-changes in biofilm mass, it was evident that biofilm of *S. aureus* cultured for 24, 48 and 72h, subsequently inoculated with *P. aeruginosa* accumulated a significantly ($P<0.05$) higher mass of biofilm (Figure 3A and B) relative to biofilm of *S. aureus* alone. Conversely, mono-species biofilms of *P. aeruginosa* inoculated with *S. aureus* as a secondary coloniser did not show any significant increase in biofilm mass relative to single-species biofilm of *P. aeruginosa* (Figure 3A and B). Of note is the statistically significant ($P<0.05$) decrease in the accumulation of biofilm for 24h established biofilm of *P. aeruginosa* following inoculation with *S. aureus* and further incubation for 24h (Figure 3A and B).

To ascertain whether increased biofilm formation of *P. aeruginosa* when inoculated onto pre-formed biofilm of *S. aureus* was a consequence of direct attachment to *S. aureus*, microtitre plates were coated with protein extracts derived from 24, 48 and 72h old biofilms of *S. aureus*. This resulted in a 2-2.6-fold increase in *P. aeruginosa* biofilm relative to *P. aeruginosa* mono-species biofilm. This effect was not observed when the *S. aureus* proteins coating the MTP were blocked with an anti-staphylococcal antibody (Figure 3C), and biofilm accumulation was significantly ($P<0.05$) reduced. Addition of spent media aspirated from *S. aureus* biofilm cultured for 24, 48 and 72h, to 24, 48 and 72h old biofilm of *P. aeruginosa* showed a similar effect to the addition of live *S. aureus* to pre-formed biofilm of *P. aeruginosa*, in that the amount of biofilm was significantly ($P<0.05$) less (Figure 4A) compared to the untreated control and biofilms treated with extracts from 48 and 722

biofilm. There was a concurrent reduction in recoverable bacteria (by CFU) (Figure 4B) from 24h *P. aeruginosa* biofilms treated with secreted extracts derived from 24h *S. aureus* biofilm indicating the possibility of biofilm dispersal or killing of biofilm bacteria by *S. aureus*.

This suggests both competitive and mutual interactions between *S. aureus* and *P. aeruginosa* during early biofilm establishment whereby *S. aureus* directly disrupts immature biofilm of *P. aeruginosa*, but once it is itself established it allows for secondary attachment of *P. aeruginosa*. Furthermore, these data support clinical observations that persons who are pre-colonised with *S. aureus* have a higher predisposition for secondary infection with *P. aeruginosa* (Ahlgren et al., 2015; Limoli et al., 2016).

S. aureus and *P. aeruginosa* do not competitively attach or adhere to human plasma proteins

Human tissue proteins such as collagen and hyaluronin can influence polymicrobial biofilm development (Biyikoglu et al., 2012; Birkenhauer et al., 2014). Co-cultured *S. aureus* and *P. aeruginosa* showed impaired adherence to immobilised fibronectin, fibrinogen and collagen, that was not statistically significant ($P > 0.05$) (less than one-fold), relative to mono-cultured bacteria (Figure 5A). Calculated competitive relative indices based on TVCs recovered for each organism, indicated that despite less attachment of both bacteria overall, *S. aureus* was more adept at adhering to immobilised proteins than *P. aeruginosa* (Figure 5B).

Soluble fibrinogen, or collagen resulted in negligible co-aggregation (Figure 5C) over the time course of the experiment; soluble fibronectin did not mediate any increase or decrease in co-aggregation relative to co-aggregation in co-aggregation

buffer alone over the time course of the experiment (Figure 5C; Figure 2A). Furthermore, analysis of the composition of bacterial aggregates by TVC, and calculated competitive relative indices, indicated little if any competition for attachment (Figure 5D) as similar numbers of each bacteria were recovered.

Therefore, these data suggest that the early interactions between *S. aureus* and *P. aeruginosa* that precede biofilm establishment and maturation are not dependent upon the host proteins fibronectin, fibrinogen or collagen; this contrasts with other biofilm forming organisms that thrive as polymicrobial communities within the human host, and rely on host extracellular matrix proteins for biofilm establishment (Rickard et al., 2003; Peters et al, 2012).

Co-infection of human keratinocytes mediates increased bacterial invasion

Given the differential capacity for attachment, aggregation and interaction using a combination of microtitre-based assay and purified human proteins, we next aimed to establish whether such interactions occurred using a human cell line to represent the skin. Using an immortalised human keratinocyte infection model infected with either *P. aeruginosa* or *S. aureus*, or both organisms together in a ratio of 1:1, we observed a statistically significant ($P < 0.05$) increase in the number of *P. aeruginosa* adhered to the cell monolayer, in co-culture than in monoculture (Figure 6A). The number of internalised, and therefore invasive, *S. aureus* were significantly higher ($P < 0.05$) in co-culture compared to mono-culture (Figure 6A). Repeat experiments using either spent media (containing secreted constituents) or cellular constituents derived from washed biofilm were undertaken; for *S. aureus* these were concerned only with invasion; for *P. aeruginosa* these were concerned only with attachment. There were statistically significant ($P < 0.05$) fold-increases in invasion of

keratinocytes by *S. aureus* (calculated relative to untreated controls) in response to both secreted and cellular material derived from *P. aeruginosa* biofilms cultured for 24, 48 and 72h. Despite being statistically significant ($P < 0.05$) relative to untreated controls, but there was no significant difference between culture times (6B). This indicates that the increased invasion could be mediated by soluble or cell-bound factors. For *P. aeruginosa* infected keratinocytes treated with secreted or cellular material derived from *S. aureus* biofilms, there was no statistically significant fold-increase in attachment irrespective of culture time (Figure 6C). This suggests that intact or live *S. aureus* are likely necessary to serve as a pioneer or bridging organism when attached to human cells.

Co-cultured bacteria impair the pro-inflammatory response of damaged keratinocytes

Having demonstrated the interaction between *S. aureus* and *P. aeruginosa* impacted upon adhesion and invasion into human keratinocytes, we then explored the effect that this might have on the pro-inflammatory response of these cells since they are the first to encounter pathogens during skin/wound infection. Treatment of immortalised human keratinocytes with extracts derived from mono- and co-cultured biofilm indicated a general trend in support of an impaired pro-inflammatory response for co-cultured biofilm, which would in turn mediate reduced neutrophil migration (Figure 7). Significantly, higher levels of IL-6 were evident for co-cultured biofilm compared to *S. aureus* mono-cultured biofilm ($P < 0.05$) but the converse was true for mono-cultured *P. aeruginosa*, suggesting collectively that co-culture resulted in an intermediate response. However, the elevated production of IL-6 compared to the untreated control (20pg ml^{-1} [untreated] compared to 210pg ml^{-1} [co-culture])

suggest that this level of IL-6 production would still favour a pro-inflammatory state, synonymous with chronic infection.

Co-cultured biofilm perpetuates a non-healing state that is no more recalcitrant than mono-cultured biofilm

The pro-inflammatory response can perpetuate a state of persistent, localised damage that impairs healing and promotes the maintenance of infection. Based on our observations of differential cytokine production in response to co-cultured biofilm, monolayers of immortalised human keratinocytes were damaged by scratching, and subsequently treated with spent supernatant or cellular material derived from mono- or co-cultured biofilm. Application of secreted biofilm components derived from co-cultured biofilm (24 and 48h) had a significant effect ($P < 0.05$) on scratch repair compared to mono-cultured biofilm of both *S. aureus* and *P. aeruginosa* (Figure 8A). Specifically healing was impaired for a greater extent by extracts derived from co-culture compared to *S. aureus* in mono-culture, but healing was impaired to a lesser extent for *P. aeruginosa* compared to mono-culture. Reminiscent of the cytokine production profiles (Figure 7), this indicated an intermediate result for co-cultured biofilm, but repair was still impaired compared to the untreated control; this supports the model of impaired healing associated with wound chronicity. Cell derived components had no significant effect on scratch repair suggesting that secreted factors such as toxins or pyocyanin facilitated the observed effect, rather than cell-structures such as LPS or LTA.

Discussion

Pre-colonisation with *S. aureus* is known to be a risk factor for colonisation with *P. aeruginosa* in patients with chronic lung infection, such as that seen in cystic fibrosis. Competition between these microorganisms within the chronic infected lung is well documented and associated with worse patient outcomes (Baldan et al., 2014; Fugere et al., 2014; Limoli et al., 2017). Information derived from the study of chronic infected wounds lags that of chronic lung infection, but it is known that both *S. aureus* and *P. aeruginosa* can co-exist within the chronic wound environment; these microorganisms are the two species most commonly isolated from such wounds (Hotterbeekx et al., 2017).

Disparate evidence has described scenarios in which these two pathogens co-exist in a co-operative or competitive manner, or alternatively that they inhabit discrete sites within the wound meaning that they are unlikely to interact (Hendricks et al., 2001; Pastar et al., 2013; DeLeon et al., 2014). The latter hypothesis is based on the observation of these bacteria in biopsies of chronic infected wounds and the former have been reliant on *in vitro* and/or *in vivo* models (Fazli et al., 2009; Hotterbeekx et al., 2017). We hypothesise that during the early phases of co-colonisation *S. aureus* and *P. aeruginosa* interact, and the distinct colonisation pattern observed in wound biopsies occurs over a long period of time, during which competition for space and nutrients, drives the population towards separation. We suggest that during those early stages of co-colonisation, bacterial interaction might mediate heightened host-damage but that over time the pathoadapted population undergoes niche partitioning with a concurrent reduction in host damage, conducive to maintenance of a stable microbial population.

The findings presented here demonstrate that a two-species biofilm comprised of *S. aureus* and *P. aeruginosa* produces a significantly ($P < 0.05$) and consistently greater biofilm accumulated over a period of 72h, and that within these biofilms *S. aureus* is most numerous as a consequence of successful competition against *P. aeruginosa*, evidenced by analyses of relative competitive indices. Examination of early co-aggregation events that precede biofilm establishment infers advantageous circumstance for *P. aeruginosa* which aggregates more effectively in the presence of *S. aureus*. Despite this, bacterial aggregation was diminished overall, but *S. aureus* was most numerous within the aggregates. Soluble human serum proteins did not enhance aggregation nor attachment, contrary to other studies which have demonstrated that collagen and hyaluronin at wound sites can influence early polymicrobial adhesive events (Birkenhauer et al., 2014). Contemporary biofilm research has described the important role of bacterial aggregates in biofilm formation, indicating that it is possible that biofilms are seeded wholly or in part from aggregates (Kragh et al., 2016; Melaugh et al., 2016). This being the case our results indicate that by co-aggregating with *S. aureus* the likelihood of colonisation by *P. aeruginosa* is elevated.

Further to these data, we demonstrate that when *S. aureus* is employed as a pioneer coloniser in biofilm growth assays it augments the attachment of *P. aeruginosa*. This effect is not reciprocal, moreover *S. aureus* added as a secondary coloniser to biofilms of *P. aeruginosa* established for 24h, results in diminished overall biofilm biomass indicating that *S. aureus* disrupts biofilm of *P. aeruginosa* in some way, possibly either by dispersal or through direct bactericidal activity. Additionally, secreted components derived from biofilm of *S. aureus* replicated this effect suggesting that biofilm dispersal relies on a secreted factor. *S. aureus*, but

not *S. epidermidis*, is known to produce nucleases that either prevent or disrupt biofilm growth for a number of bacterial pathogens, including *P. aeruginosa* (Pihl et al., 2010; Tang et al., 2011; Yang et al., 2011; Pihl et al., 2013). Furthermore, the secreted protein SpA (staphylococcal protein A) is known to interact with *P. aeruginosa* to impair biofilm formation and enhance the propensity to persist (Armbruster et al., 2016); it is therefore likely that the effects we have observed are mediated by either of these two proteins.

In a human cell model we demonstrate that *S. aureus* increases the numbers of *P. aeruginosa* that attach to the surface of keratinocytes. The same effect was not observed when keratinocytes are inoculated with *P. aeruginosa* in a suspension containing cellular components or spent media derived from staphylococcal biofilm suggesting that whole staphylococci, attached to human keratinocytes are required for augmented adhesion of *P. aeruginosa*. This synergy is reciprocal; in co-culture *P. aeruginosa* results in significantly ($P < 0.05$) larger numbers of *S. aureus* invading the keratinocyte cell line, an effect which is also observed when *S. aureus* was inoculated into the cell line in the presence of spent media or cell-derived material from *P. aeruginosa* biofilm. Synergy of virulence between *S. aureus* and *P. aeruginosa* has been previously reported but appears strain-specific with no conserved interactions described (Hendricks et al., 2001; Pastar et al., 2013; DeLeon et al., 2014; Kumar and Ting, 2015; Serra et al., 2015). These include upregulation of *hla* and *pva*, encoding α -haemlysin and Pantone-Valentine leucoidin and production of staphyloxanthin, but, to our knowledge we report for the first time, an increased propensity for invasion. Of note are the disparate consequences of interaction between *S. aureus* and *P. aeruginosa* on virulence which highlights the importance of co-adaptation between these two organisms.

Damaged or infected keratinocytes produce a number of cytokines including IL-6, IL-8 and TNF- α . Keratinocytes respond to these cytokines by proliferating and differentiating, a secondary function of these cytokines is to promote inflammation and the recruitment of macrophage to the site of infection. Co-infected keratinocytes showed an altered cytokine profile compared to single-species infection. Generally, the response was heightened when compared to *S. aureus* alone, but dampened when compared to *P. aeruginosa* alone. This intermediate response to co-infection is conducive to the maintenance of low-level tissue damage associated with chronicity, prolonged colonisation and impaired wound repair. Scratch assay corroborated these findings and those of others, in which co-culture impaired healing. For some secreted components this was statistically significant, but for cellular derived extract, healing was impaired to no greater extent than for mono-culture. Certainly, studies of intra-abdominal wound infection indicate that polymicrobial infection does not impair the rate of healing and recovery compared to mono-microbial infection (Shah et al., 2015). Conversely other studies have shown that re-epithelialisation is delayed by polymicrobial infection comprising *S. aureus* and *P. aeruginosa* (Pastar et al., 2013). During this study we were not able to establish whether the impaired healing observed was due to arrested keratinocyte proliferation or migration.

Collectively, the findings of this study indicate clear interactions between *S. aureus* and *P. aeruginosa* that are both competitive and reciprocally beneficial to each organism, in terms of pathogenicity and colonisation. Several studies have shown that these bacteria interact in polymicrobial infection resulting in differential immune response and enhanced virulence. Indeed alginate production by *P. aeruginosa* has been shown to promote co-existence, and the immune protein

calproectin induced co-colonisation of the lung by these two pathogens (Wakeman et al 2016; Limoli et al., 2017). But analysis of wound biopsies show clear segregation between the two species (Fazli et al., 2009). Therefore within the context of the broader body of research describing both *in vitro* and *in vivo* interaction between *S. aureus* and *P. aeruginosa*, we suggest that these bacteria interact early on to afford the best chance of colonisation and to augment virulence, but that once established, both facets diminish and the two bacteria separate into distinct niches. Recent work using laser electrospray ionisation mass spectrometry has indeed shown significant co-localisation between these microorganisms in biofilm cultured for 24 hours, rather than self-segregation, which might be in part due to dynamic phenotypic changes that occur in *S. aureus* during early attachment to biotic and abiotic surfaces (Williams et al., 1997; William's et al, 1999; Dean et al., 2015).

Our findings also support the pre-colonisation hypothesis that is well established for cystic fibrosis lung, whereby persons pre-colonised with *S. aureus* are pre-disposed to secondary colonisation with *P. aeruginosa*. There is an emerging international consensus that most of the current guidelines on the prevention and treatment of biofilms are of limited utility in chronic wounds as bacteria are present in biofilms as aggregated communities (Hoiby et al., 2015; WHO, 2015; WUWHS, 2016). Clinicians could prioritise patient groups who are at a higher risk of colonisation with *P. aeruginosa* by screening chronic wounds for *S. aureus* and subsequently be in a position to intervene at the earliest opportunity. Globally, diagnostic labs are equipped to routinely culture and identify *S. aureus* therefore such an intervention could be utilised in both developed and transitional

countries to help to manage the burden of infection disease and concurrently support antimicrobial stewardship.

Acknowledgements

We would like to thank the Leonardo Da Vinci program for enabling PMA to undertake this work. We would also like to thank Raymond Samuriwo for helpful insights.

References

- Ahlgren HG, Benedetti A, Landry JS, *et al.* Clinical outcomes associated with *Staphylococcus aureus* and *Pseudomonas aeruginosa* airway infections in adult cystic fibrosis patients. *BMC Pulm Med* 2015;**15**:67.
- Altoparlak U, Erol S, Akcay MN, Celebi F, Kadanali A. The time-related changes of antimicrobial resistance patterns and predominant bacterial profiles of burn wounds and body flora of burned patients. *Burns*. 2004; **30**:660–664
- Armbruster K, Wolter DJ, Mishra M, *et al.* *Staphylococcus aureus* protein A mediated intraspecies interactions at the cell surface of *Pseudomonas aeruginosa*. *MBio* 2016;**3**: e00538-16.
- Baldan R, Cigana C, Testa F, *et al.* Adaptation of *Pseudomonas aeruginosa* in Cystic Fibrosis airways influences virulence of *Staphylococcus aureus in vitro* and murine models of co-infection. *PLoS ONE* 2014;**9**:e89614.
- Barnabie PM, Whiteley M. Iron-Mediated Control of *Pseudomonas aeruginosa*-*Staphylococcus aureus* Interactions in the Cystic Fibrosis Lung. *J Bacteriol* 2015;**197**:2250–1.
- Birkenhauer E, Neethirajan S, Weese JS. Collagen and hyaluronan at wound sites influence early polymicrobial biofilm adhesive events. *BMC Microbiology* 2014;**14**:191.
- Biyikoglu B, Ricker A, Diaz, PI. Strain specific colonisation patterns and serum-modulation of multi-species oral biofilm development. *Anaerobe* 2012; **18**:459-470
- Clarke SR and Foster SJ. Surface adhesins of *Staphylococcus aureus*. *Adv Microb Physiol* 2006; **51**:187-224

Dalton T, Dowd SE, Wolcott RD, Sun Y, Watters C, Griswold JA, Rumbaugh KP. An in vivo polymicrobial biofilm wound infection model to study interspecies interactions. *PLoS One* 2011; **6**: e27317

Davies TJ. Evolutionary Ecology: when relatives cannot live together. *Curr Biol* 2006; **16**:R645–7.

Dean SN, Walsh C, Goodman H, van Hoek ML. Analysis of mixed biofilm (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) by laser ablation electrospray ionization mass spectrometry. *Biofouling* 2015; **31**:151–161.

DeLeon S, Clinton A, Fowler H, *et al.* Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an in vitro wound model. *Infect Immun* 2014; **82**:4718–28.

Dohmen PM. Antibiotic resistance in common pathogens reinforces the need to minimise surgical site infections. *J Hosp Infect* 2008; **Suppl 2**:15–20.

Fazli M, Bjarnsholt T, Kirketerp-Møller K, *et al.* Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. *J Clin Microbiol* 2009; **47**:4084–9.

Filkins LM, Graber JA, Olson DG, *et al.* Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* Drives *S. aureus* towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model. *J Bacteriol* 2015; **197**:2252–64.

Fugere A, Seguin DL, Mitchell G, *et al.* Interspecific small molecule interactions between clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from adult cystic fibrosis patients. *PLOS One* e86705.

Gottrup F, Apelqvist J, Bjarnsholt T, *et al.* Antimicrobials and Non-Healing Wounds. Evidence, controversies and suggestions-key messages. *J Wound Care* 2014; **23**:477–8.

Guggenheim M, Thurnheer T, Gmür R, Giovanoli P, Guggenheim B. Validation of the Zürich burn-biofilm model. *Burns* 2011; **37**:1125–1133

Hendricks KJ, Burd TA, Anglen JO, *et al.* Synergy between *Staphylococcus aureus* and *Pseudomonas aeruginosa* in a rat model of complex orthopaedic wounds. *J Bone Joint Surg Am* 2001; **83**:855–61.

Høiby N, Bjarnsholt T, Givskov M, *et al.* Antibiotic resistance of bacterial biofilms. *Int J Antimicrob Agents* 2010; **35**:322–32.

Høiby N, Bjarnsholt T, Moser C, *et al.* ESCMID guideline for the diagnosis and treatment of biofilm infections 2014; *Clin Microbiol Infect* 2015; **Suppl 1**:S1–25.

Hotterbeekx A, Kumar-Singh S, Herman Goossens, Malhotra-Kumar S. *In vitro* and

in vivo interactions between *Pseudomonas aeruginosa* and *Staphylococcus* spp. *Front Cell Infect Microbiol* 2017, DOI: 10.3389/fcimb.2017.00106

Kolenbrander PE, Palmer RJ Jr, Periasamy S, Jakubovics NS. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* 2010; **8**:471-480

Kragh KN, Hutchison JB, Melaugh G, *et al.* Role of multicellular aggregates in biofilm formation. *MBio* 2016;22: e00237

Kumar A, Ting YP. Presence of *Pseudomonas aeruginosa* influences biofilm formation and surface protein expression of *Staphylococcus aureus*. *Environ Microbiol* 2015;**17**:4459–68.

Limoli DH, Yang J, Khansaheb MK, *et al.* *Staphylococcus aureus* and *Pseudomonas aeruginosa* co-infection is associated with cystic fibrosis-related diabetes and poor clinical outcomes. *Eur J Clin Microbiol Infect Dis* 2016;**35**:947–53.

Limoli DH, Whitfield GB, Kitao T, *et al.* *Pseudomonas aeruginosa* alginate overproduction promotes coexistence with *Staphylococcus aureus* in a model of cystic fibrosis respiratory infection. *MBio*; e00186-17.

Maliniak ML, Stecenko AA, McCarty NA. A longitudinal analysis of chronic MRSA and *Pseudomonas aeruginosa* co-infection in cystic fibrosis: A single-center study. *J Cyst Fibros* 2016;**15**:350–6.

Mashburn LM, Jett AM, Akins DR, Whiteley M. *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during *in vivo* coculture. *J Bacteriol* 2005;**187**:554–66.

Melaugh G, Hutchison J, Kragh KN, *et al.* Shaping the Growth Behaviour of Biofilms Initiated from Bacterial Aggregates. *PLoS One* 2016;e0149683

Metcalf DG, Bowler PG. Clinician perceptions of wound biofilm. *Int Wound J* 2016;**13**:717–25.

Miles AA, Misra SS, Irwin JO. The estimation of the bactericidal power of the blood. *J Hyg (Lond)* 1938;**38**:732–49.

Pastar I, Nusbaum AG, Gil J, *et al.* Interactions of methicillin resistant *Staphylococcus aureus* USA300 and *Pseudomonas aeruginosa* in polymicrobial wound infection. *PLoS ONE* 2013;**8**:e56846.

Peters BM, Jabra-Rizk MA, O'May GA, Costerton JW, Shirtliff ME. Polymicrobial interaction: impact on pathogenesis and human disease. *Clin Microbiol Rev* 2012. **25**:193-213

Pihl M, Davies JR, Chavez P, Svensater G. Differential effects of *Pseudomonas*

aeruginosa on biofilm formation by different strains of *Staphylococcus epidermidis*. *FEMS Immunol Med Microbiol* 2010;**59**:439-446.

Pihl M, Arvidsson A, Skepo M, *et al*. Biofilm formation by *Staphylococcus epidermidis* on peritoneal dialysis catheters and the effects of extracellular products from *Pseudomonas aeruginosa*. *FEMS Path and Dis* 2013;**67**:192-198.

Phalak P, Chen J, Carlson R, Henson M. Metabolic modelling of a chronic wound biofilm consortium predicts spatial partitioning of bacterial species. *BMS Systems Biology* 2016; **10**:90

Prince A. Adhesins and receptors of *Pseudomonas aeruginosa* associated with infection of the respiratory tract. *Microb Pathog* 1992; **13**:251-260

Rasigade JP, Moulay A, Lhoste Y, *et al*. Impact of sub-inhibitory antibiotics on fibronectin-mediated host cell adhesion and invasion by *Staphylococcus aureus*. *BMC Microbiol* 2011;**11**:263.

Rhoads DD, Wolcott RD, Percival SL. Biofilms in wounds: management strategies. *J Wound Care* 2008;**17**:502–8.

Rickard AH, Gilbert P, High NJ, *et al*. Bacterial co-aggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* 2003;**11**:94-100

Serra R, Grande R, Butrico L, *et al*. Chronic wound infections: the role of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Expert Rev Anti Infect Ther* 2015;**13**:605–13.

Shah PM, Edwards BL, Dietch ZC, *et al*. Do polymicrobial intra-abdominal infections have worse outcomes than monomicrobial intra-abdominal infections? *Surg Infect* 2016;**17**:27-31.

Tang J, Kang M, Chan H, *et al*. The staphylococcal nuclease prevents biofilm formation in *Staphylococcus aureus* and other biofilm-forming bacteria. *Sci China* 2011;**9**:863-869.

Tümmler B, Wiehlmann L, Klockgether J, Cramer N. Advances in understanding *Pseudomonas*. *F1000Prime Rep* 2014;**6**:9.

Waeman CA, Moore JL, Noto MJ, *et al*. The innate immune protein calprotectin promotes *Pseudomonas aeruginosa* and *Staphylococcus aureus* interaction. *Nat Comms* 2016;**7**:11951.

Williams I, Venables WA, Lloyd D, Paul F, Critchley I. The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus*. *Microbiology* 1997; **143:2407-2413**

Williams I, Paul F, Lloyd D, *et al* Flow cytometry and other techniques show that *Staphylococcus aureus* undergoes significant physiological changes in the early stages of surface-attached culture. *Microbiology* 1999; 145:1325-1333

Woods J, Boegli L, Kirker KR, *et al*. Development and application of a polymicrobial, *in vitro*, wound biofilm model. *J Appl Micro* 2010; 112:998-1006

World Health Organisation. Global Action Plan on Antimicrobial Resistance. 2015; Available from:

http://apps.who.int/iris/bitstream/10665/193736/1/9789241509763_eng.pdf?ua=1

World Union of Wound Healing Societies. Position Document: Management of Biofilm. London: Wounds International. 2016

Yang L, Liu Y, Markussen T, *et al*. Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *FEMS Immunol Med Microbiol* 2011; 62:339-34

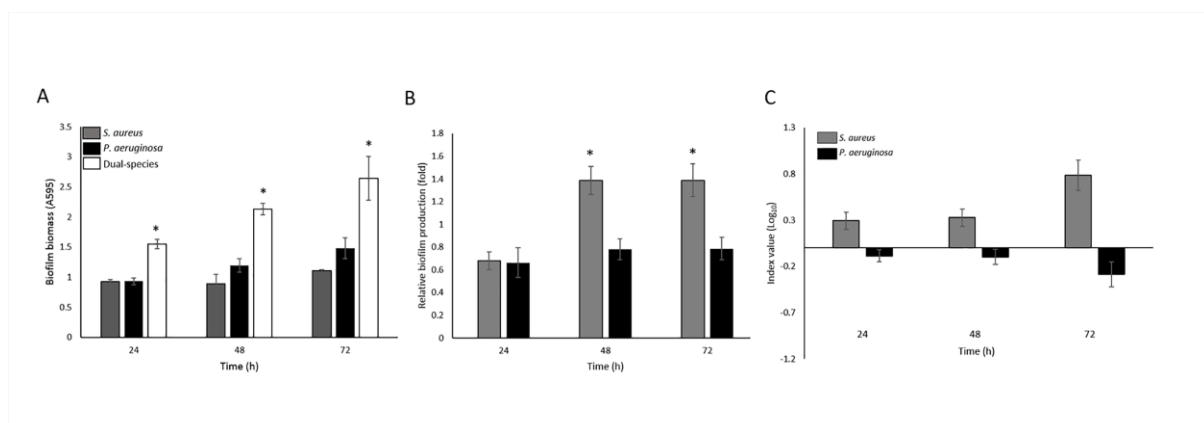


Figure 1. Biofilm growth and competition between *S. aureus* and *P. aeruginosa* in co-culture. (A) Increase in biofilm biomass accumulation for *S. aureus* and *P. aeruginosa* in mono- and co-culture (B) Biofilm accumulation expressed as a fold increase compared to each microorganism in mono-culture; * indicates significant differences where $P < 0.05$. (C) Relative competition between *S. aureus* and *P. aeruginosa* in co-cultured biofilm over 72h.

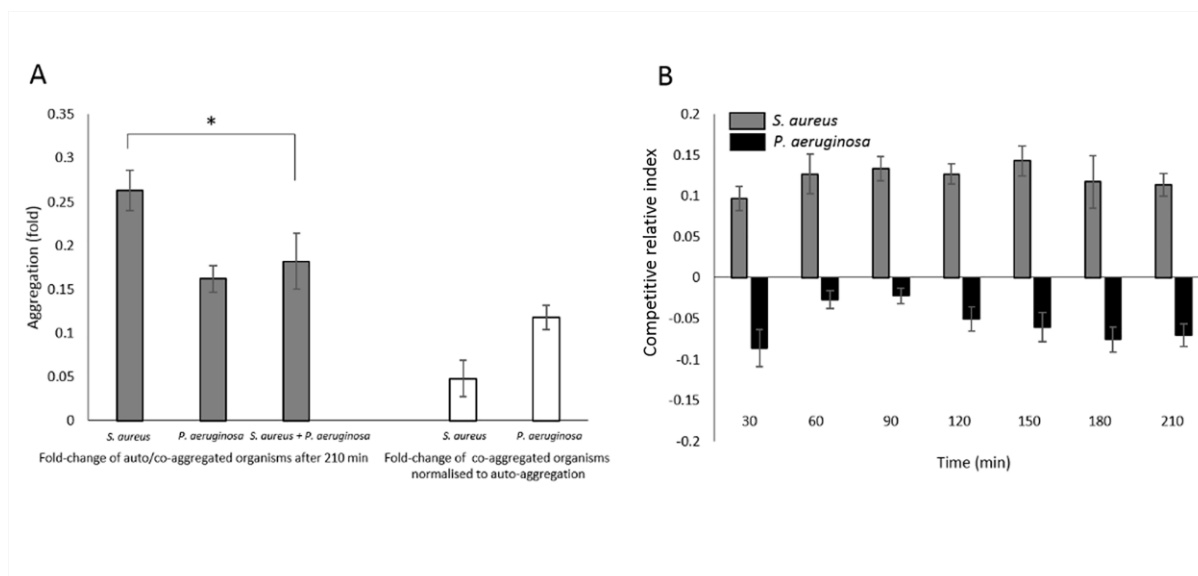


Figure 2. Auto- and co-aggregation between *S. aureus* and *P. aeruginosa*. (A) Fold change in auto- and co-aggregation after 210min (compared to t=0), and fold change between each co-aggregated organism compared to each auto-aggregated organism; * indicates significant differences where $P < 0.05$. (B) Relative competition between co-aggregated *S. aureus* and *P. aeruginosa*.

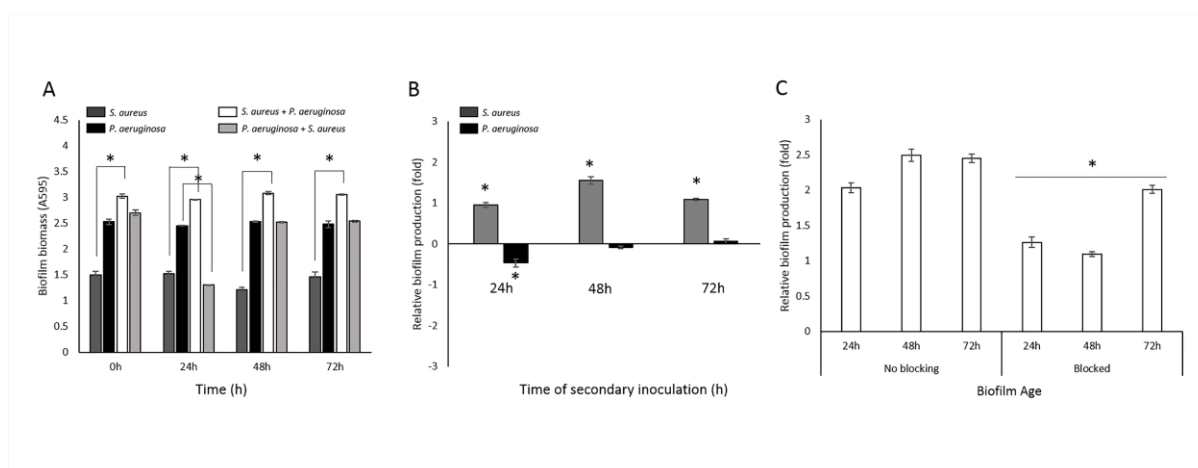


Figure 3. Biofilm growth following consecutive inoculation of primary and secondary coloniser. (A) Biofilm biomass of mono-cultured biofilm and dual-species biofilm to which bacteria were added consecutively (B) Relative biofilm accumulation of co-cultured organisms compared to mono-cultured organisms; * indicates significant differences where $P < 0.05$. (B) Relative biofilm biomass of *P. aeruginosa* when inoculated onto immobilized *S. aureus* derived biofilm components, with and without blocking; * indicates significant differences where $P < 0.05$.

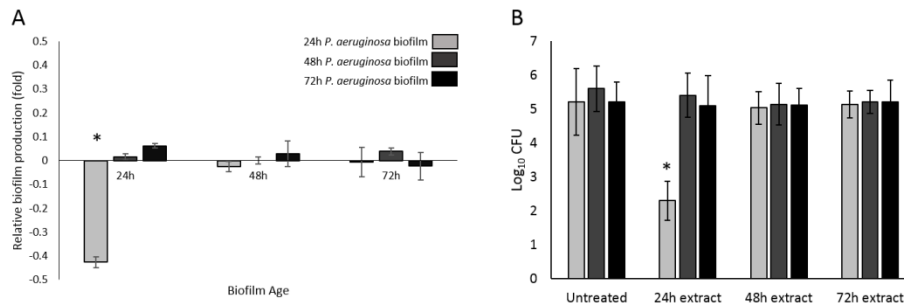


Figure 4. The effect of *S. aureus* and extracts derived from *S. aureus* biofilm on the development of *P. aeruginosa* biofilm over 72h. (A) The relative fold change in biofilm is compared to an untreated control; * indicates significant differences where $P < 0.05$. (B) Corresponding CFU counts derived from treated biofilm.

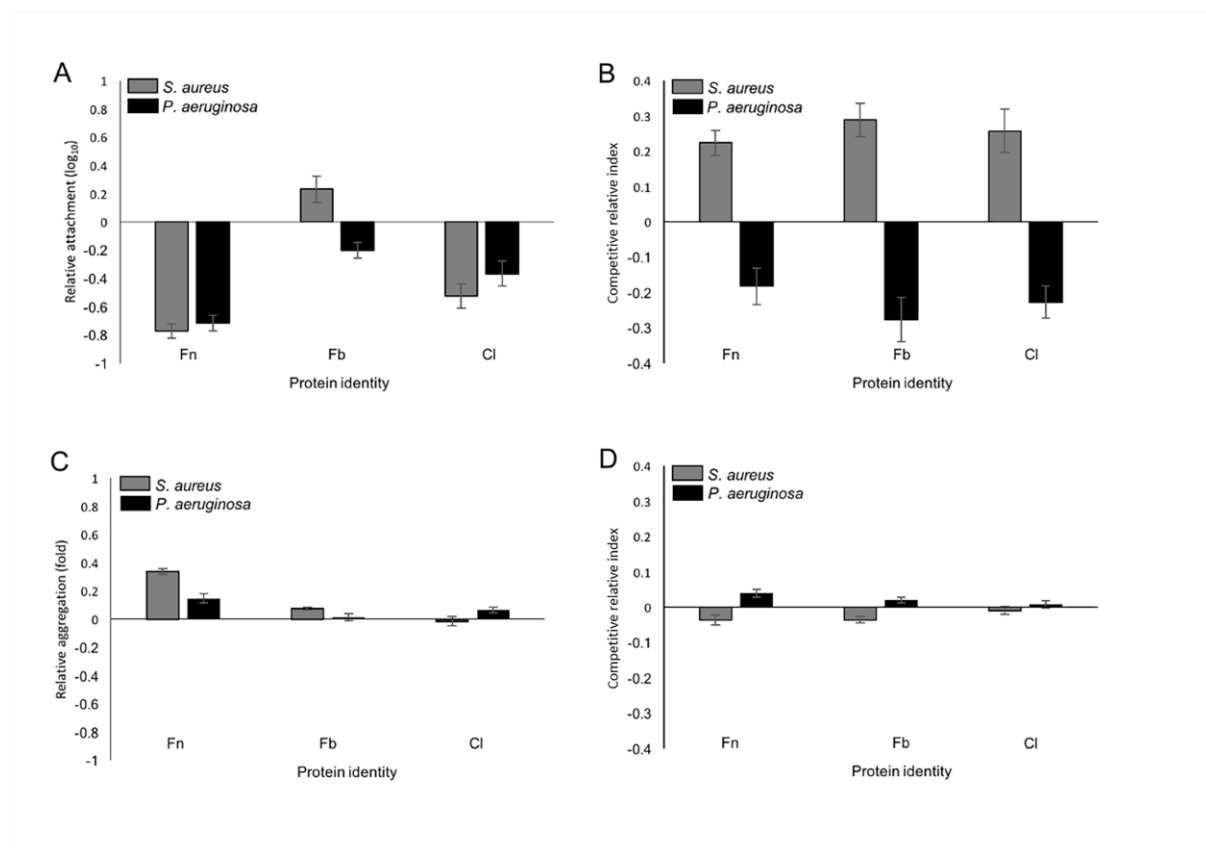


Figure 5. Attachment of *S. aureus* and *P. aeruginosa* to immobilised and soluble human tissue proteins. (A) Relative fold-change attachment to immobilised fibronectin (Fn), fibrinogen (Fb) and collagen (Cl) for each organisms in co-culture compared to mono-culture. (B) Relative competition between *S. aureus* and *P. aeruginosa* for attachment to immobilised fibronectin, fibrinogen, or collagen. (C) Relative fold-change attachment to soluble fibronectin (Fn), fibrinogen (Fb) and collagen (Cl) for each organism in co-culture compared to mono-culture. (D) Relative competition between *S. aureus* and *P. aeruginosa* for attachment to soluble fibronectin, fibrinogen, or collagen.

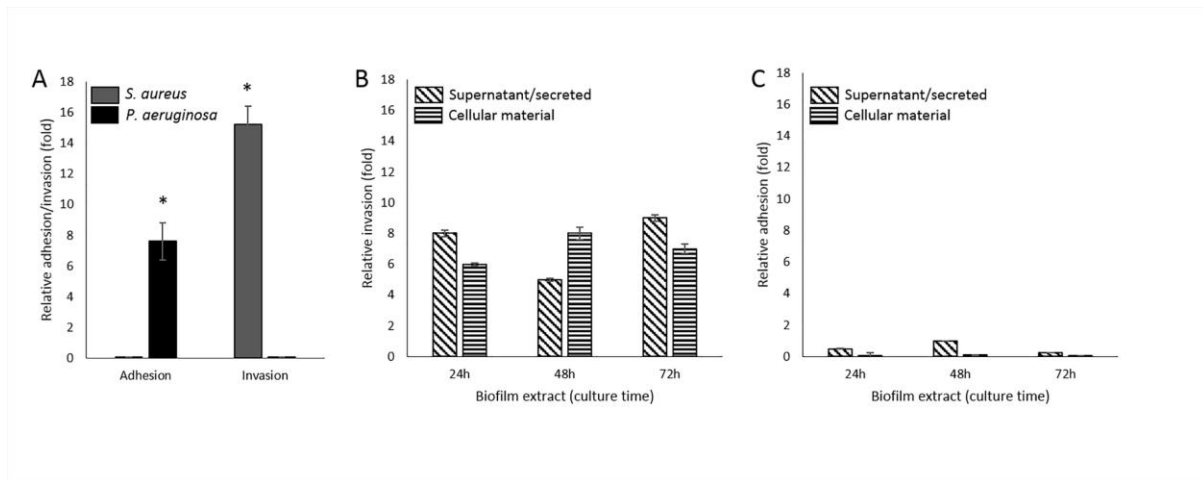


Figure 6. Adhesion and invasion of *S. aureus* and *P. aeruginosa* to human keratinocytes. (A) Relative adhesion and invasion compared to monoculture; * indicates significant differences where $P < 0.05$. (B) Relative invasion of *S. aureus* in the presence of *P. aeruginosa* biofilm extracts (24-72 h biofilm) (C) Relative attachment of *P. aeruginosa* in the presence of *S. aureus* biofilm extract (24-72h biofilm).

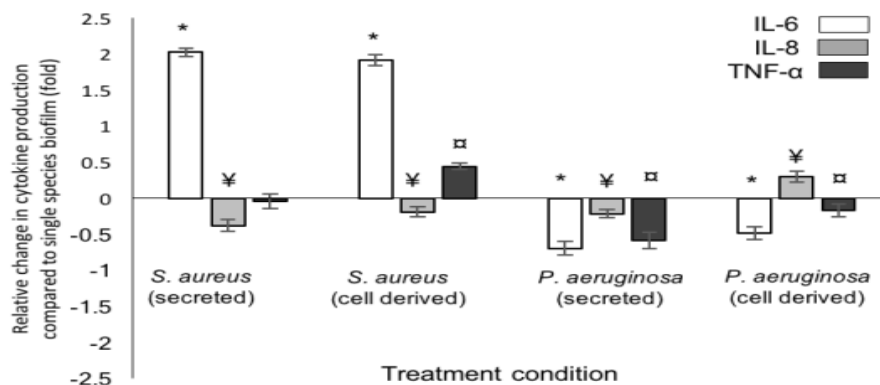


Figure 7. Relative cytokine expression profile for human keratinocytes treated with biofilm derived components (secreted or cellular) from *S. aureus* and *P. aeruginosa* co-cultured biofilm, expressed as a fold-change compared to mono-species biofilm; * denotes a significant change on the production of IL-6 ($P < 0.05$), α denotes a significant change in the production of IL-8 ($P < 0.05$), ¥ denotes a significant change in the production of TNF- α ($P < 0.05$).

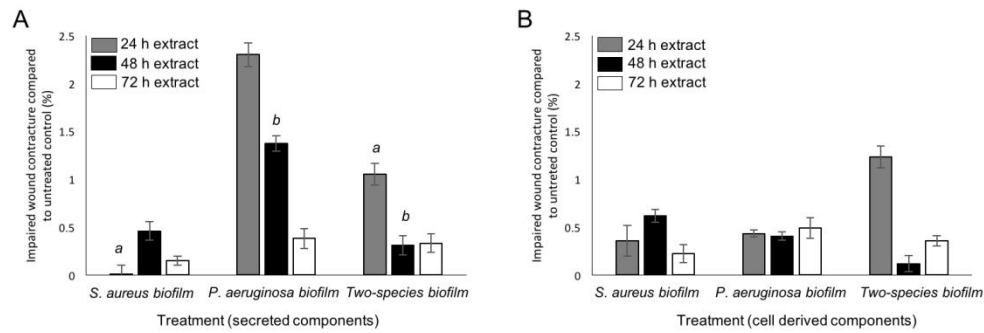


Figure 8. Wound contracture of scratched human keratinocyte monolayers in the presence of biofilm derived secreted or cellular components. (A) Secreted components from 24, 48 and 72h cultured biofilm; *a* denotes a significant difference between single species *S. aureus* and two-species biofilm ($P < 0.05$), *b* denotes a statistically significant difference between single species *P. aeruginosa* and two-species biofilm ($P < 0.05$). (B) Cellular components from 24, 48 and 72h cultured biofilm